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Stimulus-dependent phosphorylation of profilin-1 in angiogenesis

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Abstract

Angiogenesis, the formation of new blood vessels, is fundamental to development and post-injury tissue repair. Vascular endothelial growth factor (VEGF)-A guides and enhances endothelial cell (EC) migration to initiate angiogenesis. Profilin-1 (Pfn-1) is an actin-binding protein that enhances actin filament formation and cell migration, but stimulus-dependent regulation of Pfn-1 has not been observed. Here, we show VEGF-A-inducible phosphorylation of Pfn-1 at Tyr¹²⁹ is critical for EC migration and angiogenesis. Chemotactic activation of VEGF receptor kinase-2 (VEGFR2) and Src induce Pfn-1 phosphorylation in the cell leading edge, promoting Pfn-1 binding to actin and actin polymerization. Furthermore, Pfn-1 phosphorylation is robustly and preferentially elevated in blood vessels during tissue repair after myocardial infarction in humans. Conditional endothelial knock-in of phosphorylation-deficient Pfn-1^{Y129F} in mice reveals that Pfn-1 phosphorylation is critical for angiogenesis in response to wounding and ischemic injury, but not for developmental angiogenesis. Thus, VEGFR2/Src-mediated phosphorylation of Pfn-1 bypasses canonical, multistep intracellular signaling events to initiate EC migration and angiogenesis, and might serve as a selective therapeutic target for anti-angiogenic therapy.

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CONTRIBUTIONS

Y.F. designed, performed and analyzed all experiments, produced figures, and wrote the initial draft of the paper. A.A. generated and analyzed the data in Fig. 1c-e, 2e, and Fig. S4d. Y.Q. designed and performed immunohistochemistry analysis in Fig. 5a. J.J. contributed to the analysis of the Biacore in Fig. 2d. S.M.E. performed phylogenetic analysis of Pfn-1. B.W. identified Pfn-1 phosphorylation site by mass spectrometry as shown in Fig. 1b and Supplementary Fig. S2. A.H., L.M.G., and M.S.P. helped write and edit the final draft of the manuscript. P.L.F. designed, supervised, and analyzed all experiments and wrote the final draft of the manuscript.

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The authors declare no competing financial interests.

Chemotaxis is the directional movement of cells in response to chemical stimulation, and is driven by the force generated by polarized actin polymerization, in turn regulated by an ensemble of actin-binding proteins¹. Angiogenesis is the sprouting and outgrowth of new blood vessels from pre-existing vessels, and is initiated by endothelial cell (EC) migration, primarily induced by vascular endothelial growth factor (VEGF)-A²⁻⁵. Likewise, VEGF-A guides directional EC migration and capillary branching during embryogenesis⁶, and in pathological settings such as tissue remodeling after ischemia^{4,7}. Profilin-1 (Pfn-1) is an actin-binding protein, ubiquitously expressed by fungi, plants, certain viruses, and most animal cells. Pfn-1 promotes actin polymerization and cell movement by catalyzing the exchange of actin-bound ADP for ATP, and by liberating actin monomer (G-actin) from its sequestering protein, thymosin- β 4 (T β 4); both processes facilitate unidirectional addition of G-actin to elongating actin filaments⁸. *PFN1* gene disruption causes gross impairment of development and embryonic lethality in insects and mice⁹⁻¹¹. Recently, an important role of Pfn-1 in wound-induced EC motility has been reported^{12,13}. Despite the important functions of Pfn-1 in actin polymerization and cell motility, angiogenic stimulus-dependent regulation of Pfn-1 function has not been investigated.

RESULTS

VEGF-A induces phosphorylation of Pfn-1 at Tyr¹²⁹ during EC migration

Expression of lentivirus-driven shRNA targeting the 3'-UTR inhibited Pfn-1 expression in mouse aortic EC by about 80%, and migration in response to VEGF-A by about 50%, confirming the important role of Pfn-1 in VEGF-A-induced EC migration; expression of the brain-specific homologue Pfn-2 was not reduced (Fig. 1a and Supplementary Fig. S1). To investigate stimulus-dependent regulation of Pfn-1 activity, we investigated post-translational modification of Pfn-1 in migrating EC. Proteomic studies of tumor cells have revealed Pfn-1 phosphorylation of Tyr⁶⁰ and Tyr¹²⁹^{14,15}, and phosphatidylinositol 3-kinase (PI3K)-mediated Pfn-1 phosphorylation at Ser¹³⁷ by thrombin in platelets¹⁶ has been reported, but the role of these modifications in cell migration is not known. His-tagged human Pfn-1 was expressed in human umbilical vein EC, and Pfn-1 in lysates from subconfluent, migrating cells was assessed by immunoprecipitation and LC-tandem mass spectrometric analysis; 13 peptides with 72% coverage were identified. Collision-induced dissociation (CID) spectra revealed three phospho-peptides: SSFpY⁶⁰VNGLTLGGQK, TKpS⁹²TGGAPTFNVTVTk, and CpY¹²⁹EMoASHLR (Fig. 1b and Supplementary Fig. S2); other post-translational modifications, including methylation and acetylation, were not detected. Stimulus-inducible Pfn-1 phosphorylation by VEGF-A was confirmed by ³²P-labeling *in vitro*. Lysates from VEGF-A-treated, sub-confluent cells induced robust phosphorylation of purified Pfn-1, whereas lysates from confluent EC showed only weak labeling activity; Pfn-1 phosphorylation was verified by immunoprecipitation (Fig. 1c). To confirm the result *in vivo*, cells were metabolically labeled with ³²P-orthophosphate. Basal and VEGF-A-inducible Pfn-1 phosphorylation was substantially greater in sub-confluent, migrating cells (Fig. 1d).

Using phospho-specific antibodies, VEGF-A-inducible Pfn-1 phosphorylation in sub-confluent cells was detected primarily on Tyr (Fig. 1e). Multiple sequence alignment of Pfn in vertebrate species revealed conservation of Tyr¹²⁹, but not Tyr⁶⁰, suggesting the former is more likely to exhibit an important regulatory function (Fig. 1f). Immunoblot analysis with a polyclonal antibody we generated against a P-Tyr¹²⁹-containing Pfn-1 peptide confirmed VEGF-A-inducible phosphorylation of Tyr¹²⁹ in migrating EC (Fig. 1g). To confirm these results in a cellular context, EC were transfected with Flag-tagged Pfn-1, stimulated to migrate, and phosphorylation determined by immunoprecipitation and probing with anti-phospho-Tyr antibody. Phosphorylation of Y129A and Y129F Pfn-1 mutants was markedly

diminished compared to wild-type protein (Fig. 1h). Taken together, these data indicate Tyr¹²⁹ is the major VEGF-A-responsive phosphorylation site in Pfn-1.

Pfn-1 phosphorylation at Tyr¹²⁹ promotes binding to G-actin and actin polymerization

To explore the function of Pfn-1 Tyr¹²⁹ phosphorylation, binding partners of Flag-tagged Pfn-1 in migrating EC were determined by mass spectrometry. β -actin was identified as the major Pfn-1 target that preferentially binds the phosphorylated, wild-type form but not the phosphorylation-defective Y129A mutant (Fig. 2a). The stimulus- and phosphorylation-dependent interaction between β -actin and Pfn-1 was confirmed by co-immunoprecipitation (Fig. 2b). Structural and biochemical studies show Tyr¹²⁹ is in the C-terminal α -helix crucial for Pfn-1 binding to actin (Fig. 2c)¹⁷. Determination of binding constants by surface plasmon resonance showed that phosphorylation of Pfn-1 (by *in vitro* incubation of wild-type Pfn-1 with lysate from VEGF-A-activated cells) moderately increased its affinity for β -actin; similar treatment of the Y129A mutant was ineffective (Fig. 2d). Phosphorylation might enhance binding to β -actin by facilitating an electrostatic interaction between phospho-Tyr¹²⁹ in Pfn-1 and nearby Arg³⁷² in β -actin (Fig. 2c). Moreover, compared to the non-phosphorylatable Y129A mutant, phosphorylated Pfn-1 nearly doubled the initial rate of nucleotide exchange on G-actin required for addition to growing actin filaments (Fig. 2e). To determine the effect of Tyr¹²⁹ phosphorylation on actin filament formation, G-actin was induced to polymerize *in vitro* in the presence of thymosin β 4, barbed-end-free spectrin F-actin seeds, and wild-type or Y129A Pfn-1 pre-treated with lysate from VEGF-A-activated cells¹⁸. Pfn-1 liberates G-actin from thymosin β 4 and promotes its addition to the barbed-end of F-actin⁸. Phospho-Pfn-1 increased the initial rate of actin polymerization substantially compared to phosphorylation-dead Pfn-1^{Y129A} (Fig. 2f,g). Thus, stimulus-inducible phosphorylation of Pfn-1 at Tyr¹²⁹ increases its affinity for G-actin, enhances nucleotide exchange on actin, and promotes actin polymerization *in vitro*.

Several proline-rich proteins and phosphatidylinositol 4,5-bisphosphate (PIP₂) are Pfn-1 ligands, and their interaction regulates actin dynamics and cell motility^{8,19}. Co-immunoprecipitation experiments revealed that Pfn-1 phosphorylation does not significantly affect its interaction with proline-rich proteins VASP and N-WASP (Supplementary Fig. S3a). Interestingly, the 3-dimensional X-ray structure of the Pfn-1/actin/VASP ternary complex reveals that Pfn-1 Tyr¹²⁹ is close to or within the actin binding site, but on the opposite side of the protein from the domain that binds the poly-L-proline domain of VASP²⁰ (Supplementary Fig. S3b). Additionally, lipid co-sedimentation and filtration binding analyses showed that phosphorylation of Pfn-1 does not influence its binding to PIP₂ (Supplementary Fig. S3c-f).

Pfn-1 phosphorylation at Tyr¹²⁹ is induced by VEGFR2 and Src kinases

We investigated the identity of the kinase responsible for VEGF-A-induced Pfn-1 phosphorylation, initially focusing on known downstream kinases of VEGFR2, the major receptor kinase for angiogenic signaling by VEGF-A⁷. The immediate downstream tyrosine kinases of VEGFR2 include focal adhesion kinase (FAK) and Src family members Src, Fyn, and Lyn²¹. siRNA-mediated knockdown indicated that only Src contributes to VEGF-A-induced Pfn-1 phosphorylation (Fig. 3a). Moreover, pharmacological inhibitors of VEGFR2 (indolin and dihydroindol, 5 μ M), and a receptor tyrosine kinase inhibitor (PD173074, 5 μ M), almost completely inhibited VEGF-A induced Src phosphorylation at Tyr⁴¹⁶, the autophosphorylation site necessary for Src activation, suggesting VEGF-A induces VEGFR2-dependent Src activation (Fig. 3b). To examine the possibility that Src is a proximal, i.e., direct Pfn-1 kinase, cell lysates were subjected to immunodepletion and used as kinase source for *in vitro* phosphorylation. Depletion of Src markedly suppressed Pfn-1 phosphorylation (Fig. 3c). Remarkably, depletion of VEGFR2 in lysates modestly reduced

Pfn-1 phosphorylation as well. Immunoblot analysis with anti-phospho-Pfn-1-Tyr¹²⁹ antibody confirmed site-specific phosphorylation by VEGFR2 and more robust phosphorylation by Src (Fig. 3d). The kinase domain of VEGF receptor-3 (VEGFR3) that responds primarily to VEGF-C and -D, and FAK, showed low activity towards this target. An immunocomplex phosphorylation assay confirmed that VEGFR2- and Src-containing complexes in cell lysates directly phosphorylate Pfn-1 at Tyr¹²⁹ (Fig. 3e).

Direct Pfn-1 phosphorylation by VEGFR2 and its immediate downstream kinase suggests that VEGF-A may bypass intracellular signaling cascades to induce Pfn-1 phosphorylation. Consistent with this idea, pharmacological inhibition of VEGFR2 and receptor tyrosine kinases, almost completely suppressed VEGF-A-stimulated Pfn-1 phosphorylation at Tyr¹²⁹, whereas inhibitors of PI3K (LY294002, 10 μ M) and PKC (calphostin C, 2 μ M) were ineffective (Fig. 3f). Moreover, PI3K-independence of VEGF-A-induced Pfn-1 phosphorylation was shown by the absence of inhibition by siRNA-mediated knockdown of major PI3K isoforms implicated in vascular development and angiogenesis, namely, p110 α , β , γ , and δ (Fig. 3g). Likewise, expression of constitutively active, myristoylated Akt, a major effector kinase of PI3K, did not stimulate Pfn-1 phosphorylation (Fig. 3h), confirming PI3K-independent Pfn-1 phosphorylation by VEGF-A.

Ligand and receptor specificity was shown by the absence of robust Pfn-1 phosphorylation by other growth factors, including the VEGFR1-specific agonist placenta growth factor (PlGF), VEGF-C, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and sphingosine-1-phosphate (S1P) (Fig. 3i,j). Interestingly, an inhibitory peptide that targets the VEGF-A binding site of VEGFR1, and knockdown of VEGFR1, inhibited VEGF-A-induced Pfn-1 phosphorylation (Supplementary Fig. S4a,b), suggesting that VEGFR1 contributes to Pfn-1 phosphorylation. Although VEGFR1 exhibits *in vitro* kinase activity directed to Pfn-1 (Supplementary Fig. S4c-e), ectopic expression of VEGFR2, but not VEGFR1, effectively reconstituted VEGF-A-induced Pfn-1 phosphorylation in VEGFR-negative fibroblasts (Supplementary Fig. S4f,g). These results are consistent with a role for VEGF-binding activity, but not kinase activity, of VEGFR1 in Pfn-1 phosphorylation. Furthermore, fibroblast expression of VEGFR2 mutated at Tyr⁹⁵¹ in the docking site for TSAd/Src²², but not at Tyr¹¹⁷⁵ in the activation site for PLC γ /PI3K²³, abrogated VEGF-induced Pfn-1 phosphorylation (Supplementary Fig. S4h). Mutation of Tyr¹²¹⁴, a potential binding site for Cdc42 and Grb2²¹, also inhibited phosphorylation, suggesting a possible role of these factors in VEGFR2 activation and Pfn-1 phosphorylation. Together, these findings reveal both VEGFR2 and its downstream Src kinase as proximal, Pfn-1-selective tyrosine kinases.

Pfn-1 phosphorylation at Tyr¹²⁹ is spatially restricted to cell leading edge and is required for VEGF-A-stimulated EC migration

Polarized signals initiating cell motility generally evoke a spatially-restricted response. To visualize the spatial distribution of phosphorylated Pfn-1, EC were induced to migrate toward a VEGF-A gradient in a modified Zigmond chamber. Tyr¹²⁹-phosphorylated Pfn-1 preferentially was observed at the leading edge, almost precisely co-localizing with F-actin (Fig. 4a; Supplementary Fig. S5a,b). Transfection of cells with pECFP-Pfn-1 and detection by fluorescence showed a small amount of Pfn-1 at the leading edge in most cells with the preponderance localized far behind this region (Supplementary Fig. S5a). The spatially restricted phosphorylation of Pfn-1 is likely due to asymmetric activation of VEGFR2 and Src, as indicated by localization of Tyr⁹⁵¹-, Tyr¹¹⁷⁵-, and Tyr¹²¹⁴-phosphorylated VEGFR2 and Tyr⁴¹⁶-phosphorylated Src at the cell leading edge (Fig. 4b,c). To investigate the role of Pfn-1 phosphorylation in VEGF-A-induced EC migration, endogenous Pfn-1 was depleted by stable expression of lentivirus-driven shRNA targeting the 3'-UTR, and Pfn-1 restored by transfection with cDNA-encoding wild-type or mutated mouse Pfn-1 CDS lacking the

3'-UTR (Fig. 4d). Depletion of Pfn-1 reduced EC migration in response to VEGF-A by about 70%. Expression of wild-type Pfn-1 substantially restored motility, but phospho-dead mutants were completely ineffective. Thus, Pfn-1 phosphorylation at Tyr¹²⁹ has a critical role in VEGF-A-induced *in vitro* EC migration.

Pfn-1 phosphorylation at Tyr¹²⁹ is elevated in endothelium of infarcted heart

We have explored the *in vivo* role of Pfn-1 phosphorylation in angiogenesis during tissue repair after myocardial infarction in which VEGF-A expression enhances and directs EC sprouting from capillaries, initiating angiogenesis, tissue growth, and repair^{3,4}. Robust angiogenesis was observed in infarcted cardiac tissue from human subjects, as indicated by increased number of CD31⁺ vessels compared to normal heart (Fig. 5a). Phospho-Pfn-1-Tyr¹²⁹ was similarly elevated in infarcted tissue, co-localizing almost exclusively with CD31⁺ blood vessels. In contrast, the distribution of total Pfn-1 was uniformly expressed throughout cardiac tissue, as was the phospho-Akt-Ser⁴⁷³ product of PI3K activation. EC-specific Pfn-1 phosphorylation was confirmed by co-localization with CD144 (VE-cadherin)- and VEGFR2-positive cells (Fig. 5b,c). Quantitative immunofluorescence analysis of an array of normal and infarcted myocardium specimens showed Pfn-1 phosphorylation in infarcted tissue was 13- and 18-fold higher in acute myocardial infarction and scar-containing tissues, respectively, compared to normal heart tissue (Fig. 5d). An even greater 27-fold higher Pfn-1 phosphorylation was observed in healing granulation tissue characterized by robust angiogenesis. These results reveal a remarkable, EC-selective phosphorylation of Pfn-1 during cardiac tissue repair, and suggest a role of Pfn-1 phosphorylation in post-injury angiogenesis.

Pfn-1 phosphorylation at Tyr¹²⁹ is critical for VEGF-A-induced EC migration and post-injury angiogenesis *in vivo*

To rigorously test the role of Pfn-1 phosphorylation in angiogenesis *in vivo*, we generated conditional, EC-specific, phosphorylation-deficient Pfn-1^{Y129F} knock-in (Tie2-Cre;Pfn1^{flox/flox;Y129F}) mice in the C57Bl/6 background. The mice have the terminal exon of the Pfn1 gene, which contains the phosphorylation site, flanked with LoxP sites followed by a minigene of the exon carrying a Y129F mutation (Fig. 6a). The mice express Cre DNA recombinase under control of the EC-specific Tie2 promoter. EC-specific knock-in by Tie2-directed Cre expression was verified by sequence analysis of Pfn-1 mRNA from isolated EC and from de-endothelialized aorta in mice homozygous for the conditional null allele (Fig. 6a and Supplementary Fig. S6a). Efficient expression of the mutant Y129F Pfn-1 gene in knock-in mice was shown by near-complete abrogation of VEGF-inducible Pfn-1 phosphorylation at Tyr¹²⁹ in EC (Fig. 6b and Supplementary Fig. S6b). The Y129F mutation did not affect Pfn-1 mRNA or protein expression (Fig. 6a and Supplementary Fig. 6c), and did not alter basal EC motility (Fig. 6c) or proliferation (Fig. 6d). However, knock-in of Pfn-1^{Y129F/Y129F} inhibited VEGF-A-stimulated planar migration of EC from mouse aorta, microvessel outgrowth in aortic ring explants, and EC chemotaxis, all by about 50% (Fig. 4c,e,f). Inhibition of PI3K with the selective inhibitors LY294002 (10 μ M) and wortmannin (0.1 μ M) also reduced migration of wild-type mouse EC by 50%, but dual inhibition of PI3K and Pfn-1 phosphorylation (in Pfn-1^{Y129F/Y129F} EC) reduced chemotaxis to near the basal level (Fig. 6f). Finally, expression of constitutively active RhoA^{Q63L} and myristoylated Akt-1 did not rescue cell motility in Pfn-1^{Y129F/Y129F} EC, consistent with the PI3K-independent role of Pfn-1 phosphorylation in VEGF-A-induced EC migration (Supplementary Fig. S7).

Unexpectedly, Pfn-1 phosphorylation did not interfere with developmental angiogenesis, as indicated by apparently normal embryos with unaffected microvessel density in brain and heart at embryonic day E14.5, and intact retinal angiogenesis at postnatal day P5, in

Pfn-1^{Y129F/Y129F} knock-in mice (Fig. 7a). However, EC knock-in of Pfn-1^{Y129F/Y129F} substantially delayed wound closure after skin punch (Fig. 7b), and significantly reduced the number of microvessels within the healing wounds and larger blood vessels in the region surrounding the injury (Fig. 7c). In a separate model, angiogenesis after ischemia induced by femoral artery ligation, as determined by microvessel density in hind limbs, was reduced by more than 50% in Pfn-1^{Y129F/Y129F} knock-in mice (Fig. 7d). Pfn-1^{Y129F/Y129F} knock-in markedly suppressed blood flow recovery in the limbs (Fig. 7e), suggesting an additional role of Pfn-1 phosphorylation in arteriogenesis, i.e., collateral vessel formation. Likewise, angiography by micro-CT showed much less macrovessel growth in the ischemic limb of Pfn-1^{Y129F/Y129F} knock-in mice (Fig. 7f). These results were confirmed in a distinct model in which Pfn-1^{Y129F/Y129F} was expressed by tamoxifen-inducible, Cre-mediated recombination under control of the EC-specific Chd5 (VE-cadherin) promoter (Fig. 7g), and similar reductions in blood flow recovery and microvessel density were observed (Fig. 7h,i). Thus, Pfn-1 phosphorylation is required for post-ischemia angiogenesis and arteriogenesis, in which EC migration and vessel sprouting play a critical role. Together, these findings reveal a critical role for Pfn-1 phosphorylation in pathological angiogenesis during tissue repair after injury, but not in physiological angiogenesis during development.

DISCUSSION

Eukaryotic cells respond to spatial heterogeneities in extracellular chemoattractants including VEGF by polarized activation of chemotactic receptors²⁴, asymmetric recruitment and activation of a branching, multistep pathway initiated by PI3K/Akt and PTEN, and consequent Rho GTPase-directed actin polymerization at the cell leading edge^{25,26}. VEGF-inducible recruitment and activation of PI3K/Akt at the cell leading edge is thought to be particularly critical for EC polarization during chemotaxis²⁷. However, abrogation of PI3K activity, including the major EC isoform, p110 α , only partially inhibits VEGF-induced EC migration and vascular sprouting^{27,28}, suggesting that VEGFR might induce actin-based cell movement via an unidentified PI3K-independent pathway. Our results identify a PI3K-independent mechanism for regulating cell motility *via* stimulus-inducible phosphorylation of Pfn-1 at Tyr¹²⁹ and consequent increased interaction with G-actin. Dual activation of Pfn-1 phosphorylation and PI3K-mediated signaling are both required for maximal pro-migratory responses as suggested by the incomplete inhibition of EC migration observed when either PI3K activity or Pfn-1 phosphorylation is defective, but near-complete inhibition when both pathways are blocked. Furthermore, Pfn-1 phosphorylation by VEGFR2 and Src kinase bypasses classic multistep signaling events, thereby providing a more direct link between growth factor-induced receptor activation and actin polymerization (Fig. 7j). The intracellular signaling-independent “short-cut” by VEGFR2 phosphorylation of Pfn-1 might contribute to rapid initiation of cell polarity and determination of the migratory vector in response to extracellular VEGF stimulus. Activation of downstream tyrosine kinases, e.g., Src, likely enhances and maintains Pfn-1 phosphorylation, especially after internalization and degradation of VEGFR kinases following ligand activation²¹. Interestingly, although Pfn isoforms are present in all eukaryotes including yeast and plants, the appearance of the Tyr¹²⁹ phospho-site during evolution coincides with the appearance of vertebrates that exhibit closed circulatory systems, EC linings, and VEGFR2, suggesting that stimulus-dependent regulation of Pfn activity is essential for blood vessel homeostasis (Table S1).

Angiogenesis, initiated by directed EC migration, requires spatiotemporal regulation of cellular processes for which Pfn-1 phosphorylation may represent a critical control node. Pfn-1 phosphorylation stimulates the interaction between Pfn-1 and G-actin that is critical for EC motility and capillary morphogenesis¹⁹. Likewise, Pfn-1 phosphorylation contributes importantly to post-injury angiogenesis, as revealed by our conditional murine knock-in

model. The robust expression of VEGFR2 in vascular endothelium likely contributes to highly selective Pfn-1 phosphorylation in EC. In contrast, PI3K is required for developmental and pathological angiogenesis²⁹, and knockout of p110 α , the major PI3K isoform in EC partially inhibits cell motility but causes embryonic lethality in mouse²⁷, consistent with the widespread role of PI3K in cell growth, proliferation, survival, and motility in multiple cell types³⁰. The fractional contribution of Pfn-1 phosphorylation to VEGF-induced EC motility and vessel sprouting might complement PI3K-mediated signals to induce maximal angiogenesis, particularly during pathological states. In summary, our results reveal a regulatory system for EC migration and angiogenesis. Its EC-preferential induction in infarcted heart, but dispensability in development, suggest that Pfn-1 phosphorylation may present a highly selective and non-noxious target for therapeutic intervention of pathological angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

VEGF-A induces phosphorylation of Pfn-1 at Tyr¹²⁹ during EC migration. **(a)** Mouse aortic EC were transduced with lentivirus expressing shRNAs targeting the Pfn-1 3'-UTR (Genbank NM_011972) or control CDS of *Photinus pyralis* luciferase (Luc, Genbank M15077). Immunoblot of EC lysates with anti-Pfn-1, -Pfn-2, and -GAPDH antibodies (left). EC were subjected to Transwell migration assay with or without VEGF-A in the lower chamber (mean $\pm$ SEM, 3 experiments) (right). **(b)** Human umbilical vein EC were transfected with pTandem-Pfn-1-His or vector, and lysates were precipitated with Ni²⁺-beads, resolved in SDS-PAGE, and stained with GelCode Blue (inset). Phosphorylation sites

in Pfn-1 band were identified by mass spectrometry. The collision-induced dissociation (CID) spectrum for pTyr¹²⁹-containing peptide, CpYEMoASHLR ($m/z = 632.0$) is shown. The y_7 and y_8 ions exhibit a mass difference consistent with pTyr at position 129. **(c)** Confluent, quiescent or subconfluent, migrating bovine aortic EC were serum-deprived and treated with VEGF-A. Cell lysates were incubated with purified Pfn-1 in buffer containing γ -³²P-ATP. Proteins before and after immunoprecipitation with anti-Pfn-1 antibody were resolved by SDS-PAGE, bands visualized by autoradiography and quantified by densitometry (mean $\pm$ SEM, $n = 3$), and immunoblotted (IB) with anti-Pfn-1. **(d)** VEGF-A-treated EC were metabolically labeled with ³²P-orthophosphoric acid and cell lysates immunoprecipitated with anti-Pfn-1 antibody. Pfn-1 phosphorylation was visualized by SDS-PAGE and autoradiography, and immunoprecipitated Pfn-1 was detected by immunoblot. **(e)** EC lysates were immunoprecipitated with anti-Pfn-1 antibody, and immunoblotted with anti-phospho-Tyr, anti-phospho-Ser, anti-phospho-Thr, and anti-Pfn-1 antibodies. **(f)** Multiple sequence alignment of Pfn-1. **(g)** Phospho-specific antibody was generated by immunizing rabbits with KKCpYEMASHLRR peptide. Serum-deprived bovine aortic EC were treated with VEGF-A, and lysates resolved by SDS-PAGE, and immunoblotted with anti-P-Pfn-1-Tyr¹²⁹ and anti-Pfn-1 antibodies. **(h)** Human umbilical vein EC were transfected with pcDNA vector or pcDNA-Pfn-1-Flag with or without mutations. Cell lysates were immunoprecipitated with anti-Flag antibody, resolved by SDS-PAGE, and immunoblotted with anti-phospho-Tyr and -Pfn-1 antibodies.

Figure 2.

Pfn-1 phosphorylation at Tyr¹²⁹ promotes binding to actin and actin polymerization. (a) Bovine aortic EC were transfected with pcDNA (vector) or pcDNA-Pfn-1-Flag constructs. Cell lysates from subconfluent, migrating cells were immunoprecipitated with anti-Flag antibody, followed by SDS-PAGE and GelCode blue staining, and proteins identified by mass spectrometry. (b) EC transfected with mutant and wild-type Pfn-1 were incubated with VEGF-A, and the interaction between Pfn-1 and β-actin determined by co-immunoprecipitation. (c) Structure of human Pfn-1 by heteronuclear NMR spectroscopy (MMDB ID: 57094, NCBI) and bovine Pfn-1:β-actin by X-ray crystallography (MMDB ID: 52665, NCBI). (d) Purified Pfn-1^{WT}-His and Pfn-1^{Y129A}-His were *in vitro* phosphorylated with lysate from VEGF-A-treated EC and re-purified with Ni²⁺-beads. Left, protein was resolved by SDS/PAGE and stained with GelCode blue. Right, K_D of binding to immobilized β-actin was determined by SPR. (e) Nucleotide exchange of ATP/εATP on G-

actin (10 μ M) was induced in the presence or absence of *in vitro* phosphorylated Pfn-1^{WT}-His or Pfn-1^{Y129A}-His (1 μ M) and monitored by fluorescence at 410 nm. **(f,g)** Pfn-1-His was *in vitro* phosphorylated with lysate from VEGF-A-treated EC, and re-purified with Ni²⁺-beads. **(f)** Protein was resolved by SDS/PAGE and stained with GelCode blue. **(g)** Barbed end pyrenyl-actin polymerization was induced by adding spectrin-actin seeds (30 ng/ml) and MgCl₂ (2 mM) in G-buffer in the presence or absence of Pfn-1 and thymosin β 4 (left, 2 μ M, right, 4 μ M). Fluorescence intensity was measured with excitation at 365 nm and emission at 407 nm.

Figure 3.

Phosphorylation of Pfn-1 at Tyr¹²⁹ by VEGFR2 and Src (a) Human microvascular EC were transfected with siRNA targeting Src, Fyn, Lyn, FAK, or luciferase (Luc), and incubated with VEGF-A, and analyzed by immunoblot. (b) Human EC were pretreated with 0.1% DMSO vehicle, VEGFR2 inhibitors (dimethy-(ethoxycarbonyl)pyrrol-yl) methylidenyl indolin and bromo-(tetrahydro-1H-indolylmethylene)-dihydroindol, or receptor tyrosine kinase inhibitor (PD173074), and then incubated with VEGF-A. Tyr⁴¹⁶ phosphorylation of Src was determined by immunoblot. (c) Lysates from VEGF-A-treated human EC were immunodepleted with anti-VEGFR2 and anti- Src antibodies and then immunoblotted with the same antibodies. Pfn-1 was incubated with the depleted lysates and phosphorylation detected by immunoblot with anti-P-Pfn-1^{Y129} antibody. (d) Pfn-1 was incubated with Src, FAK and cytoplasmic domain of VEGFR2 and VEGFR3, and phosphorylation determined

by immunoblot with anti-P-Pfn-1^{Y129} antibody. **(e)** Cell lysates from VEGF-A-treated EC were immunoprecipitated with anti-VEGFR2 or anti-Src antibody or control IgG. Immunocomplexes were incubated with purified Pfn-1 in kinase assay buffer with 5 μ Ci γ -³²P-ATP. Protein was resolved by SDS-PAGE and detected by immunoblot. Phosphorylation was determined by autoradiography and immunoblot. **(f)** EC were pretreated with the LY294002 (PI3K inhibitor), calphostin C (PKC inhibitor), VEGFR2 inhibitors dimethy-(ethoxycarbonyl)pyrrol-yl)methylidenyl indolin and bromo-(tetrahydro-1*H*-indolylmethylene)- dihydroindol, or PD173074 (receptor tyrosine kinase inhibitor), and then with VEGF-A. P-Pfn-1-Tyr¹²⁹ and GAPDH were determined by immunoblot. **(g)** EC were transfected with siRNA targeting luciferase (Luc), p110 α , p110 β , p110 δ , or p110 γ , treated with VEGF-A, and analyzed by immunoblot. **(h)** EC were transduced with retrovirus encoding myristoylated (myr)-Akt-1, treated with VEGF-A, and analyzed by immunoblot. **(i, j)** EC were treated with 10 ng/ml VEGF-A, and **(i)** 50 ng/ml PlGF, and 10 ng/ml VEGF-C, or both, and with **(j)** 10 ng/ml EGF, 20 ng/ml PDGF, or 0.1 μ M sphingosine-1-phosphate (S1P). Cell lysates were analyzed by immunoblot.

Figure 4.

Pfn-1 Phosphorylation at Tyr¹²⁹ is spatially restricted to the leading edge of migrating EC, and is required for VEGF-A-induced EC migration. (a-c) EC were exposed to VEGF-A in a modified Zigmond chamber. Pfn-1-Tyr¹²⁹ and Pfn-1 were visualized by immunofluorescence and F-actin with Alexa-350 phalloidin. Arrow indicates migration direction. (b) Cells were immunostained with anti-P-VEGFR2 and anti-VEGFR2 antibodies, and visualized with Alexa 488-IgG and Alexa 568-phalloidin. (c) Cells were immunostained with anti-P-Src-Tyr⁴¹⁶ and -Src antibodies, and visualized with Alexa 488-IgG. (d) Mouse EC were transduced with lentivirus expressing shRNA targeting Pfn-1 3'-UTR or Luc

control, selected with puromycin, and transfected with pTriEx vector or pTriEx-Pfn-1 with or without mutations. Pfn-1 expression was determined by immunoblot (left). Right, VEGF-A-induced chemotaxis was determined by Transwell assay (mean $\pm$ SEM, 3 experiments) (right).

Figure 5.

Pfn-1 phosphorylation at Tyr¹²⁹ is expressed preferentially in infarcted cardiac blood vessels. **(a,b)** Human heart sections retrieved from autopsy specimens from subjects with myocardial infarcts and normal heart controls were probed with **(a)** anti-P-Pfn-1-Tyr¹²⁹, anti-CD31, anti-Pfn-1 and anti-P-Akt^{S473} antibodies, and with **(b)** anti-CD144, anti-VEGFR1, anti-VEGFR2, anti-Pfn-1, and anti-P-Akt-1-Ser⁴⁷³ antibodies. **(c)** Quantitative analysis of co-localization of P-Pfn-1-Tyr¹²⁹ and P-Akt-Ser⁴⁷³ with CD31-positive EC (mean $\pm$ SEM, $n = 5$ and 8 for localization of P-Akt-Ser⁴⁷³ and P-Pfn-1-Tyr¹²⁹, respectively). **(d)** P-Pfn-1-Tyr¹²⁹ in heart sections from healthy controls ($n = 10$), or cardiac

patients (n = 40 total) with pathological diagnosis of acute MI, granulation tissue, and scar-containing tissue, was quantified by immunofluorescence using anti-P-Pfn-1-Tyr¹²⁹ antibody.

Figure 6.

Knock-in of phospho-deficient Pfn-1^{Y129F/Y129F} in mouse EC reduces VEGF-A-induced cell migration. **(a)** Mice were generated with conditional knock-in of Pfn-1^{Y129F} driven by Cre-Lox DNA recombination (Cre-Rec) under the control of the Tie2 EC-specific promoter. Mouse aortic EC were isolated and subjected to RT-PCR analysis of Pfn-1 mRNA followed by DNA sequencing. GAPDH and total Pfn-1 were detected by immunoblot. **(b)** EC isolated from *Pfn1*^{flox/flox:Y129F} (–Tie2-Cre) and Tie2-Cre;*Pfn1*^{flox/flox:Y129F} (+Tie2-Cre) mice were treated with 10 ng/ml VEGF-A, and subjected to immunoblot analysis. **(c)** EC isolated from mice were subjected to wound-induced cell migration assay in the presence or absence of 10

ng/ml mouse VEGF-A (Mean $\pm$ SEM, 2 experiments, n = 5 for each). **(d)** Mouse aortic EC were cultured in medium containing 5% FBS and cell number determined by MTT-based assay (mean $\pm$ SEM, n = 5). **(e)** Aortic rings were implanted in Matrigel in the presence or absence of VEGF-A, and sprouting vessels quantified (mean $\pm$ SEM, n = 5). **(f)** Mouse aortic EC were pretreated with PI3K inhibitor LY294002 or wortmannin and induced to migrate in response to VEGF-A in transwell dishes (mean $\pm$ SEM, n = 6).

Figure 7.

Pfn-1 phosphorylation at Tyr¹²⁹ is required for angiogenesis during post-injury tissue repair but not for developmental angiogenesis. **(a-f)** angiogenesis analysis using *Pfn1*^{flox/flox:Y129F} mice with or without expression of Tie2-Cre. **(a)** Left: Mouse embryos at E14.5 were imaged and tissues probed with anti-CD31 antibody. Right: Retinas were dissected from mouse pups at P5 and stained with isolectin B4. **(b,c)** Punch wounds were induced in mouse skin (n = 5). **(b)** Representative images of wounds (top) and quantification of wound area (bottom, mean ± SEM). **(c)** Inner skin flap of wounds was imaged, and the wound sections probed with anti-CD31 antibody. Representative images (top) and quantification of vessel

density (bottom, mean $\pm$ SEM) on day 7 after injury. **(d-f)** Hindlimb ischemia was induced in mice. **(d)** Hindlimb sections were stained with anti-CD31 antibody. Representative images (left) and vessel density by CD31 staining (right, mean $\pm$ SEM, $n = 5-11$) on day 28 after injury. **(e)** Left: laser Doppler images. Right: perfusion rate of blood flow in ischemic hindlimb as the ratio of that in control limb (mean $\pm$ SEM, $n = 5$). Arrows indicate ischemic hindlimb. **(f)** Vasculature of ischemic limb on day 28 after injury visualized by micro-CT angiography. Arrows indicate femoral artery (FA, pink) and ligation sites (LS, blue). **(g-i)** *Cdh5-Cre/ERT;Pfn^{flox/flox}:Y129F* mice were injected with tamoxifen or PBS. **(g)** Mouse aortic EC were treated with VEGF-A, and analyzed by immunoblot. **(h,i)** Hindlimb ischemia was induced by ligation of formal artery in mice. **(h)** Perfusion rate of blood flow in ischemic hindlimb (bars indicate mean, $n = 5$). **(i)** Hindlimbs were excised on day 28 after injury, sections stained with anti-CD31 antibody, and microvessel density quantified (mean $\pm$ SEM, $n = 5$). **(j)** Schematic of Pfn-1 phosphorylation pathway in VEGF-A-induced EC migration.